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Alpha-Mannosidase-II Deficiency Results in Dyserythropoiesis and Unveils an Alternate Pathway in Oligosaccharide Biosynthesis

Daniel Chui,* Masayoshi Oh-Eda,† Yung-Feng Liao,‡ Krishnasamy Panneerselvam,† Anita Lal,‡ Kurt W. Marek,* Hudson H. Freeze,† Kelley W. Moremen,‡ Michiko N. Fukuda,† and Jamey D. Marth*

*Howard Hughes Medical Institute
and Division of Cellular and Molecular Medicine
University of California San Diego
La Jolla, California 92093
†The Burnham Institute
10901 North Torrey Pines Road
La Jolla, California 92037
‡Department of Biochemistry
University of Georgia
Athens, Georgia 30602

Summary

Alpha-mannosidase-II (αM-II) catalyzes the first committed step in the biosynthesis of complex asparagine-linked (N-linked) oligosaccharides (N-glycans). Genetic deficiency of αM-II should abolish complex N-glycan production as reportedly does inhibition of αM-II by swainsonine. We find that mice lacking a functional αM-II gene develop a dyserythropoietic anemia concurrent with loss of erythrocyte complex N-glycans. Unexpectedly, nonerythroid cell types continued to produce complex N-glycans by an alternate pathway comprising a distinct α-mannosidase. These studies reveal cell-type-specific variations in N-linked oligosaccharide biosynthesis and an essential role for αM-II in the formation of erythroid complex N-glycans. αM-II deficiency elicits a phenotype in mice that correlates with human congenital dyserythropoietic anemia type II.

Introduction

Vertebrate cell surfaces are covered with a diverse and dynamic repertoire of asparagine-linked (N-linked) oligosaccharides (also termed N-glycans, indicating linkage to underlying protein). Complex N-glycans are the most abundant type found on the cell surface, and alpha-mannosidase-II (αM-II) acts as a key enzyme in their biosynthesis by catalyzing the first committed step in the conversion of hybrid to complex forms (Kornfeld and Kornfeld, 1985; Schachter, 1991; Figure 1). In the Golgi apparatus, αM-II cleaves two mannose residues attached in α3 and α6 linkages from the hybrid N-glycan $GlcNAc_1Man_5GlcNAc_2$-Asn, thereby producing a processed hybrid that is also the specific substrate of GlcNAc-TII (Harpaz and Schachter, 1980; Tulsiani et al., 1982; Moremen et al., 1994; Figure 1). This role is further supported from studies of the Ric15 BHK cell line, in which a reduction in αM-II activity occurs with an attenuation of complex N-glycan production (Hughes and Feeney, 1986). An exogenous inhibitor of αM-II activity known as swainsonine is found in plants of the genus Swainsona (Elbein et al., 1981; Tulsiani et al., 1982). Ingestion by vertebrates produces a disease known as "locoism," biochemically similar to α-mannosidosis and associated with aberrant behavior, male sterility, cytoplasmic vacuolation, and the accumulation of hybrid N-glycans in the brain and other tissues (Dorling et al., 1978; Colgate et al., 1979; Tulisani et al., 1988). In animal studies with the purified alkaloid, the effects of locoweed ingestion appear to be due to swainsonine (Tulsiani et al., 1984, 1988). However, a role for αM-II deficiency in these responses is not clear, since swainsonine is also a potent inhibitor of the lysosomal α-mannosidase, which functions in N-glycan catabolism (Dorling et al., 1980; Tulsiani et al., 1982; Tulsiani and Touster, 1987).

Human deficiency of αM-II has been reported in one case of congenital dyserythropoietic anemia (CDA) type II, also known as HEMPAS (hereditary erythroblastic multinuclearity with a positive acidified serum-lysis test) disease (Crookston et al., 1969; Fukuda et al., 1990). CDA type II/HEMPAS is inherited in an autosomal recessive manner, with patients developing mild-to-severe anemia associated with splenomegaly and marrow erythroplasia in the presence of multinucleated erythroblasts (reviewed in Fukuda, 1993). Most patients live a normal lifespan without neurologic involvement, although complications, including hepatomegaly with cirrhosis, hemosiderosis, gallstones, and diabetes, frequently develop. CDA type II erythrocytes commonly exhibit plasma membrane abnormalities with loss of complex N-linked oligosaccharides from glycoproteins band 3 (*AE1*) and band 4.5 (*GLUT1*) (Baines et al., 1982; Scartezzini et al., 1982; Fukuda et al., 1984, 1992). However, the clinical diagnosis of CDA type II encompasses heterogenic symptoms with only one reported case thus far associated with αM-II deficiency, in which the patient retained only 10% of normal αM-II levels with an unresolved genetic defect.

The biological role of αM-II is further puzzling, considering its pivotal position in complex N-glycan production and the relatively mild phenotype of CDA type II when compared to GlcNAc-TII deficiency in the next biosynthetic step. Studies have linked inactivating mutations in the *MGAT2* gene to human carbohydrate-deficient glycoprotein syndrome (CDGS) type II (Tan et al., 1996). This autosomal recessive disease presents severe symptoms early with children exhibiting failure to thrive, dysmorphic features, severe mental retardation, and susceptibilty to multiple infections (Jaeken et al., 1994; Charuk et al., 1995). In order to understand how αM-II functions in mammalian physiology and in N-glycan diversification, it was necessary to inactivate the *αM-II* gene in the mouse germline. We report that mice lacking a functional *αM-II* allele develop a dyserythropoietic anemia similar to CDA type II, with production of abnormal erythrocytes lacking complex N-glycans. Unexpectedly, complex N-glycan production continued among nonerythroid cell types in the absence of αM-II activity by an alternate biosynthetic pathway.

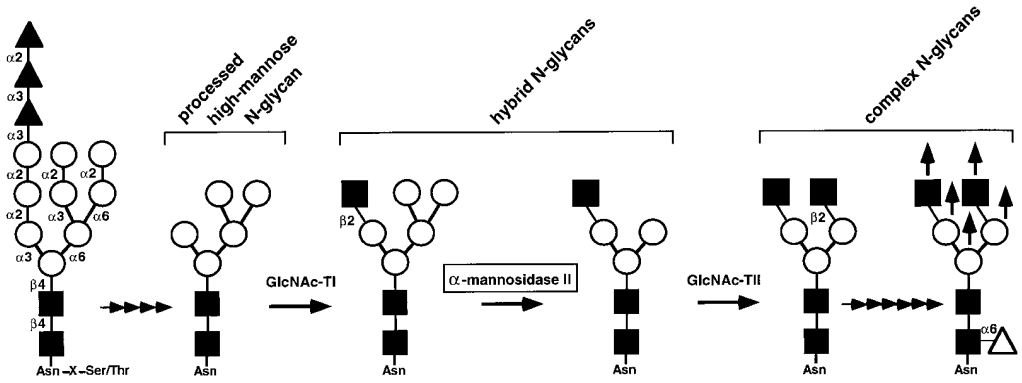

Figure 1. Asparagine-Linked Oligosaccharide Biosynthesis

The depicted oligosaccharide precursor is transferred from lipid to asparagines on nascent peptides in the endoplasmic reticulum prior to glucosidase and α1–2 mannosidase trimming. In the medial Golgi, *Mgat1*-encoded GlcNAc-TI initiates hybrid N-glycan synthesis. Conversion to complex N-glycans requires α-mannosidase-II to generate the processed hybrid N-glycan that is also the substrate of GlcNAc-TII. Anomeric linkage types are denoted. Closed triangles represent glucose; open circles, mannose; closed squares, N-acetylglucosamine. Addition of fucose (open triangle) may occur earlier than indicated. Multi-antennary complex N-glycans result from other branching reactions (arrows).

Results

α*M-II* Gene Inactivation in Embryonic Stem Cells and Mice

αM-II is a type II transmembrane protein of 1150 amino acids encoded by a single gene locus in studied vertebrates (Moremen and Robbins, 1991; Moremen et al., 1994). A mouse genomic clone that contained an exon representing a portion of the lumenal "stem" domain of αM-II was isolated. As sequence analysis indicated that excision of this exon would create a translational frameshift mutation, a gene targeting construct was produced that would permit excision of this exon and removal of the marker genes by Cre recombinase (Figure 2A; reviewed in Marth, 1996). G418 resistant embryonic stem cell clones were analyzed for homologous recombination events, producing a modified α*M-II* locus bearing three *loxP* sites as required to produce systemic (Δ) and conditional (F) mutations (Figures 2B and 2C). Chimeric mice generated from ES clones 34–107 and 31–107 were bred to C57BL/6 mates to produce mice heterozygous for $\alpha M\text{-}II^{\Delta}$ or $\alpha M\text{-}II^{F}$ alleles, respectively. Offspring homozygous for either allele were obtained at normal frequencies, and those bearing $\alpha M\text{-}II^{\Delta}$ alleles were further analyzed.

Mice Homozygous for the $\alpha M\text{-}II^{\Delta}$ Allele Lack αM-II Enzyme Activity

αM-II activity can be specifically determined using the fluorescent substrate $GlcNAc_1Man_5GlcNAc$-pyridylamine (-PA). Release of mannose alters the chromatographic mobility of this molecule in a defined manner. αM-II has been immunolocalized to the early Golgi apparatus in most cell types (Moremen et al., 1994), and Golgi fractions from genotyped mouse tissues were examined for αM-II activity by a 2 to 24 hr incubation with substrate in the presence or absence of swainsonine. αM-II activity was present in wild-type samples with the generation of the cleaved products $GlcNAc_1Man_4GlcNAc$-PA and $GlcNAc_1Man_3GlcNAc$-PA, but was absent from extracts derived from $\alpha MII^{\Delta}/\alpha M\text{-}II^{\Delta}$ mice or extracts treated with swainsonine (Figures 3A and 3B; data not shown). Additionally, an antibody specific for αM-II failed to immunoprecipitate αM-II activity from total cell extracts derived from $\alpha MII^{\Delta}/\alpha M\text{-}II^{\Delta}$ samples (data not shown). These experiments demonstrated that the exon deletion produced in the α*M-II* gene ($\alpha M\text{-}II^{\Delta}$) results in a complete loss of enzyme activity and is thus a genetic null allele.

Mice Lacking a Functional α*M-II* Allele Exhibit Splenomegaly Associated with Dyserythropoietic Anemia

Mice lacking a functional α*M-II* allele did not display noticable neurologic or behavioral symptoms and bred normally with either wild-type or homozygous-null mates. Histologic findings of the liver, brain, kidney, lymph nodes, heart, lung, and pancreas were unremarkable. Leukocyte development, morphology, circulation, and colonization of secondary lymphoid organs also appeared normal in αM-II-deficient mice (data not shown). However, analyses of peripheral blood indicated high levels of reticulocytes and significant variations in erythrocyte cell size (anisocytosis) (Figure 4A). αM-II-deficient mice of all ages invariably exhibited a marked splenomegaly (Figure 4B). Additionally, detailed hematologic analyses revealed a mild-to-moderate anemia, accompanied by a marked anisocytosis, reduced osmotic fragility, indicating a likely increase in the surface area to volume ratio, reticulocytosis involving up to 30% of circulating red blood cells, and increased levels of the i-antigen (Figures 4C-4G). The αM-II-null bone marrow exhibited normal cellularity but an increase in erythroblasts (Figure 4H). Anisocytosis, reduced osmotic fragility, increased i-antigen levels, and erythroblast hyperplasia were observed in αM-II-null mice of all ages, whereas the severity of the anemia and reticulocytosis increased with age.

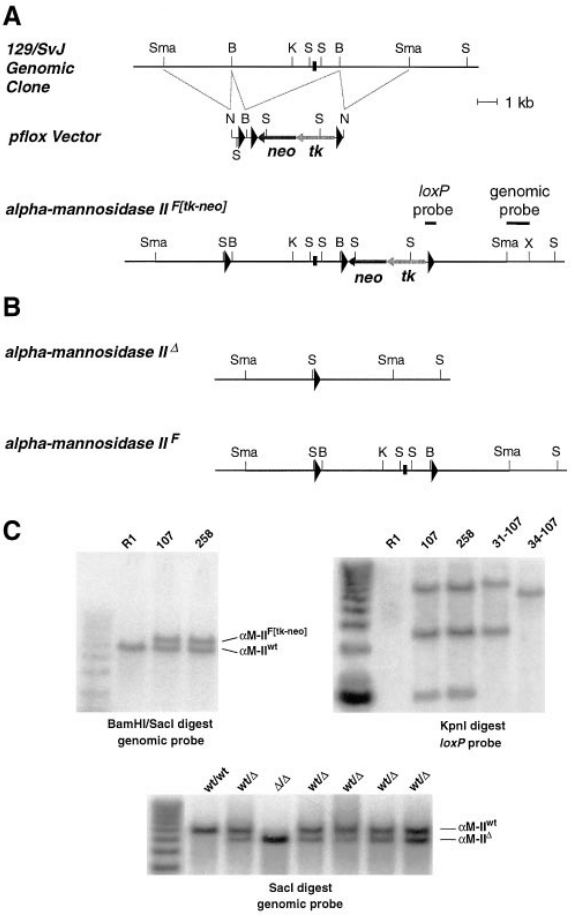

Figure 2. αM-II Gene Mutagenesis in Embryonic Stem Cells and Mice

(A) A mouse genomic clone of αM-II bearing a single exon (closed box) was isolated and used in constructing the αM-II gene targeting vector with pflox as indicated. Homologous recombination with wild-type embryonic stem cells (R1) generates the F[tk-neo] allele.

(B) Transient Cre recombinase expression and ganciclovir selection will result in ES subclones bearing αM-II$^{\Delta}$ (systemic-null) and αM-II^{F} (conditional-null) alleles.

(C) Genomic Southern blotting confirmed the expected αM-II allelic structures. Upper left: targeted ES clones 107 and 258 harbored the 7.5 kb (F[tk-neo]) allele and the 6.5 kb wild-type (wt) allele. Upper right: clone 107 bearing all three *loxP* sites was used in producing cells bearing α*M-II*^{F} (31–107) and α*M-II*$^{\Delta}$ (34–107) alleles. Lower panel: mice heterozygous and homozygous for the 6.5 kb α*M-II*$^{\Delta}$ allele are shown from Southern blot analyses of tail DNA. One-kilobase molecular weight ladders are in left lanes of above. Restriction enzyme sites Sma, SmaI; B, BamHI; K, KpnI; S, SacI; X, XbaI are denoted.

Complex N-Glycan Deficiency in Mutant Erythrocyte Membranes

A normal profile of membrane-associated and cytoskeletal proteins was observed in αM-II-deficient erythrocytes by SDS-PAGE and Coomassie blue staining, indicating that no changes occurred in the expression of the major membrane constituents (Figure 5A, left panel). Glycoproteins bearing complex N-linked oligosaccharides can be directly demonstrated by binding to the lectin E-PHA (Yamashita et al., 1983; Kobata and Endo, 1992). This binding requires the action of GlcNAc-TII, which cannot occur without αM-II (Figure 1). In wild-type erythrocytes, at least six glycoproteins bearing complex N-linked oligosaccharides were visualized by E-PHA binding (Figure 5A, right panel). This binding was absent from mutant samples, indicating that αM-II deficiency abrogates the production of complex N-glycans in erythrocytes. Mouse erythrocyte glycoproteins bearing complex N-linked oligosaccharides appeared upon close inspection to be distinct from those observed by Coomassie blue.

Erythroid Cells from αM-II-Null Mice Are Uniquely Deficient in Complex N-Glycans

Unexpectedly, analyses of various hematopoietic cells with E-PHA-biotin revealed that only erythocytes were deficient in complex N-glycans (Figure 5B). Thymocytes, myeloid cells, and mature lymphocytes displayed essentially normal levels of E-PHA binding, while erythroblast cell surfaces in the bone marrow were variably deficient. O-glycosylation appeared unaffected, as expected (Figure 5C); however, E-PHA histochemistry of the liver, kidney, spleen, pancreas, epidermis, and brain also indicated a normal level of cell surface complex N-glycans on αM-II-null cells (data not shown). Surprisingly, congenital loss of αM-II function appeared to ablate complex N-glycan production specifically in the erythroid lineage. This implied that an undisclosed alternate biosynthetic pathway was operating in other cell types. The nature of this alternate pathway was investigated by analyzing N-linked oligosaccharide biosynthesis and structural diversity in αM-II-deficient cells that retained E-PHA binding.

Structural Analyses of N-Glycan Biosynthesis in the Absence of αM-II

Splenocytes and primary fibroblasts were metabolically labeled with glucosamine or mannose for various times prior to isolating N-linked oligosaccharides for structural studies, using chromatography and exoglycosidase digestions. Complex N-glycan production in the absence of αM-II could occur from an altered lipid-linked oligosaccharide precursor lacking four mannose residues normally added by Dol-P-Man (Stoll et al., 1982). However, HPLC analyses did not reveal such an altered precursor in αM-II-deficient cells (Figure 6A). Newly synthesized oligosaccharide precursors are sometimes rapidly cleaved intact from lipid and protein; however, the proportion and size of those liberated during the labeling were also the same in control and mutant cells (data not shown). These results indicate that αM-II-null cells synthesize a normal lipid-linked oligosaccharide precursor and transfer this to protein as efficiently as wild-type cells.

ConA affinity chromatography is useful in determining the proportion of tetra-, tri- and biantennary complex, hybrid, and high mannose N-linked oligosaccharides (Baenziger and Fiete, 1979; Narasimhan et al., 1979). Surprisingly, αM-II-deficient cells contained N-linked oligosaccharides that eluted with the ConA-Sepharose

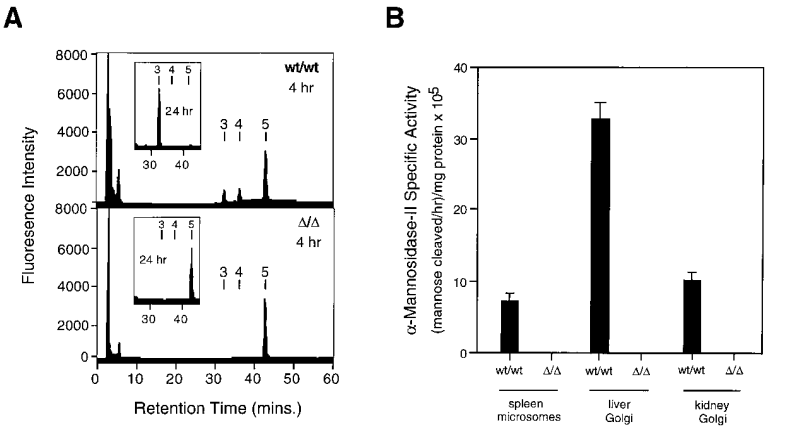

Figure 3. Absence of αM-II Activity in Mice Homozygous for the αM-II$^{\Delta}$ Allele

(A) HPLC profile following 4 or 24 hr incubation of $GlcNAc_1Man_5GlcNAc$-PA with microsomal spleen membrane extracts, demonstrating no detectable processing in the αM-II$^{\Delta}$/αM-II$^{\Delta}$ sample (lower panel). Retention time of oligosaccharide standards are indicated at the top of each panel: 3, $GlcNAc_1Man_3GlcNAc$-PA; 4, $GlcNAc_1Man_4GlcNAc$-PA; 5, $GlcNAc_1Man_5GlcNAc$-PA.

(B) Spleen, liver, and kidney derived from αM-II$^{\Delta}$/αM-II$^{\Delta}$ mice lacked αM-II activity. Shown are specific activity measurements after incubation with $GlcNAc_1Man_5GlcNAc$-PA for 24 hr as in (A). The rate of mannose cleavage was determined in the linear range of the cleavage assay and calculated as a percentage of the total susceptible mannose residues cleaved per hour. Shown are mean values and SEM from three separate comparative experiments with individual animals.

flowthrough and in response to 10 mM α-methyl glucoside, as characteristic of complex forms. These were, however, distinctly underrepresented in αM-II-deficient extracts (Figure 6B, peaks a, b, e, and f; data not shown). The proportion of high mannose N-glycans was unaltered, and hybrid N-glycans were significantly increased as expected (Figure 6B, peaks c, d, g, and h).

To resolve further the N-linked oligosaccharide species separated by ConA-Sepharose chromatography, fractions were desialylated and analyzed by HPLC (Figure 6C). Among those that eluted with 10 mM α-methyl-mannosidase, the abundance in peak 7 was greatly elevated with αM-II deficiency. Exoglycosidase studies of peak 7 confirmed that this was a hybrid-type N-linked oligosaccharide species (Figure 6C legend; Experimental Procedures). Similar analyses of putative complex N-linked oligosaccharides previously eluted with 10 mM α-methyl-glucoside (Figure 6C, panels b and f, peak 2) revealed the standard biantennary complex oligosaccharide NA2 (Experimental Procedures). The biantennary complex N-linked oligosaccharide in HPLC peak 2 was also confirmed by sequential exoglycosidase digestion to harbor the trimannosyl core (Figure 6D, left panels). Complex N-linked oligosaccharides bearing the trimannosyl core were similarly revealed among αM-II-deficient primary fibroblasts (Figure 6D, right panels).

These studies indicated that loss of αM-II activity did not alter levels of high mannose N-glycans but resulted in a 3-fold increase in the abundance of hybrid N-glycans. Surprisingly, complex N-glycans were present in αM-II-deficient cells at approximately 50% the level found in wild-type cells and at apparently normal levels on the cell surface (Table 1; Figure 5B).

An Alternate Pathway to Complex N-Glycan Production

The synthesis of complex N-glycans as determined above in αM-II-deficient cells was not compatible with the previously defined biosynthetic pathway. To explain these results, it was necessary to invoke the presence of a distinct αM that functions early in N-glycan biosynthesis. Moreover, given the well-defined substrate specificities of GlcNAc-TI and GlcNAc-TII, this distinct αM enzyme was predicted to be specific for $Man_5GlcNAc_2$-Asn, generating $Man_3GlcNAc_2$-Asn, which is also a substrate for GlcNAc-TI from in vitro studies (Schachter, 1986, 1991). We therefore tested Golgi and microsomal fractions of various cell types for the presence of this αM activity.

An αM activity toward $Man_5GlcNAc$-PA was found in all mouse cell and tissue types tested, including splenocytes, liver, kidney, and primary fibroblasts. This enzyme activity (termed αM-III) was optimal at pH 6.0–6.5 and, in the presence of Co^{2+}, generated $Man_4GlcNAc$-PA, $Man_3GlcNAc$-PA, and even $Man_2GlcNAc$-PA and $Man_1GlcNAc$-PA at later time points (Figure 7 and data not shown). No change in αM-III activity was observed among αM-II-deficient cells. αM-III activity in the mouse appeared identical to an αM activity previously discovered in the rat liver (Bonay and Hughes, 1991; Bonay et al., 1992) and was also greatly enriched in Golgi and microsomal preparations. A revision to the N-glycan biosynthetic pathway was therefore indicated, in which complex N-linked oligosaccharides are produced by two mutually exclusive routes (Figure 8).

Discussion

Alpha-Mannosidase Function in Complex N-Glycan Biosynthesis

Our findings are consistent with αM-II acting specifically to process the $GlcNAc_1Man_5GlcNAc_2$-Asn hybrid during N-glycan biosynthesis. In the absence of αM-II, this hybrid N-glycan is modified by Golgi β1–4 galactosyltransferase prior to a 3-fold accumulation observed in splenocytes and primary fibroblasts. Unexpectedly, complex N-glycans were found at approximately 50% of normal total cellular levels among αM-II-deficient splenocytes and fibroblasts. The initial rate of conversion to complex N-glycans was found to be unchanged, and little or no complex N-glycan deficiency could be detected on the cell surface. αM-II-null cells generated complex N-linked oligosaccharides bearing the trimannosyl core originating from an unaltered dolichol oligosaccharide precursor. These observations indicated the presence of a distinct αM activity functioning in an alternate pathway

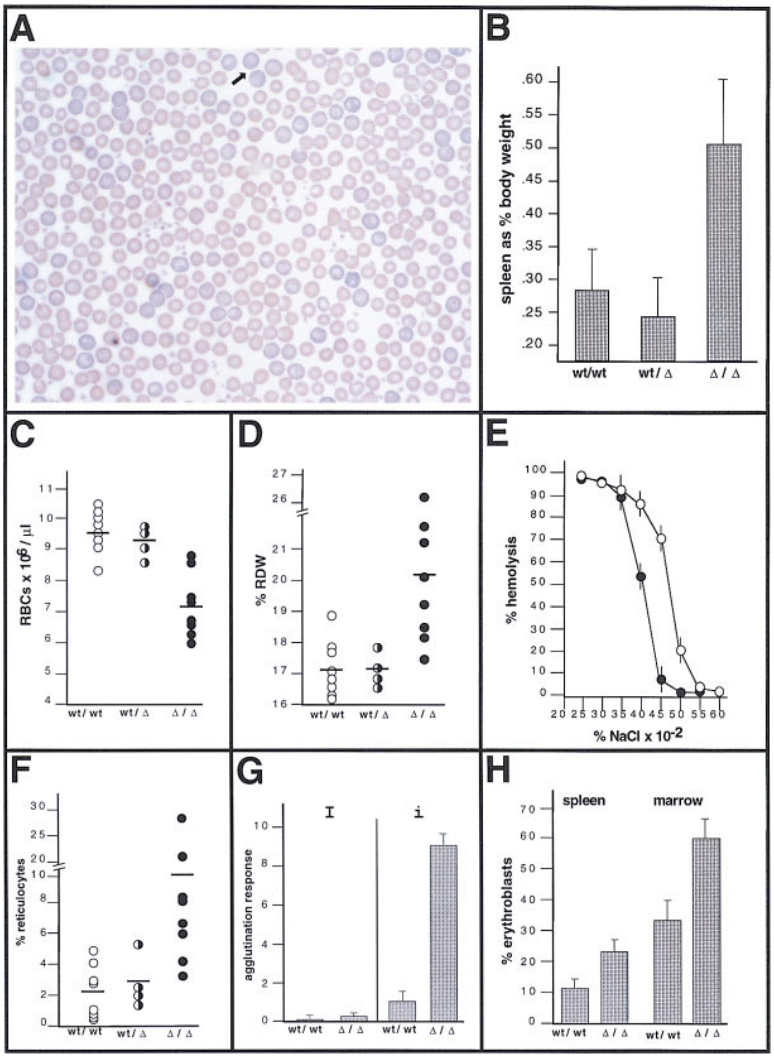

Figure 4. Dyserythropoietic Anemia in αM-II-Null Mice

(A) Peripheral blood smear stained with Wright-Giemsa from an αM-II-null mouse reveals erythrocyte cell size heterogeneity (anisocytosis) and "stress" reticulocytes (arrow).
(B) Splenomegaly was found in all αM-II-deficient mice (n = 12).
(C) Absence of αM-II resulted in a mild-to-moderate anemia.
(D) Red cell distribution width (RDW) was increased in αM-II-null mice, confirming the anisocytosis apparent in blood smears.
(E) Osmotic fragility was reduced in the absence of αM-II.
(F) Reticulocyte levels were elevated in αM-II-null mice, as detected using new methylene blue.
(G) Increased i-antigen in αM-II deficiency. Erythrocytes of either genotype failed to agglutinate with a 1/120 dilution of anti-I antisera; however, anti-i antisera agglutinated αM-II-null erythrocytes at a 1/3840 dilution as compared with a 1/480 dilution required for wild-type erythrocytes. Agglutination response to anti-i antisera was defined as the maximum-fold reduction in antibody titer that continued to produce erythrocyte agglutination (n = 3).
(H) Erythroblast frequencies in the spleen and bone marrow were increased in αM-II-null mice (n = 12).
Mean values are denoted by horizontal bars. Standard deviations from the mean are shown by vertical bars. All mice were 6 weeks of age or older and analyzed with littermate genotypic controls. Each point in (C), (D), and (F) represents a result from an individual animal. Littermate controls were included.

in complex N-glycan biosynthesis. They may also indicate a low turnover rate for complex N-glycans at the cell surface, variable transport rates to the plasma membrane, and recycling mechanisms from the membrane allowing further oligosaccharide maturation.

An αM activity acting on $Man_5GlcNAc$-PA and generating $Man_3GlcNAc$-PA (termed αM-III) was found among all mouse tissues surveyed and at levels comparable to wild-type and αM-II-null cells. αM-III activity in the mouse did not act on hybrid structures, including $GlcNAcMan_5GlcNAc$-PA in the presence of Co^{2+}, and exhibited a reduced sensitivity to inhibition by swainsonine when compared with αM-II (data not shown). Regarding swainsonine inhibition of N-glycan biosynthesis, such studies have often reported finding various levels of complex N-glycans remaining. Our analyses of αM-II-deficient mice suggest that swainsonine-induced locoism with behavioral abnormalities, male sterility, and cellular vacuolation is not a result of αM-II inhibition. Instead, swainsonine likely induces these effects by inhibition of the lysosomal α-mannosidase and perhaps other αM enzymes, depending upon the plasma levels of swainsonine attained in vivo. The alternate pathway to complex N-glycan production hence includes a relatively swainsonine-resistant αM-III activity, functioning independently and exclusive of αM-II (Figure 8). This revised pathway is also consistent with the lack of hybrid and complex N-glycans with GlcNAc-TI deficiency (Ioffe and Stanley, 1994; Metzler et al., 1994). The previous unavailability of cells bearing αM-II-null alleles in combination with the cation dependence of αM-III activity in vitro may explain why the alternate pathway was not revealed in earlier studies.

Dyserythropoiesis in Murine αM-II Deficiency and Relevance to Human CDA type II

Erythroid cells are uniquely susceptible to alterations in N-linked oligosaccharide biosynthesis evoked by αM-II deficiency. This susceptibility arises from the apparent absence of an alternate pathway in complex N-glycan production. Loss of complex N-linked oligosaccharides and other membrane-bound constituents can lead to defects involving ion transport, glycoprotein clustering, membrane fluidity, and cytoskeletal stability (Fukuda, 1993; Palek, 1995; Peters et al., 1996). An increase in

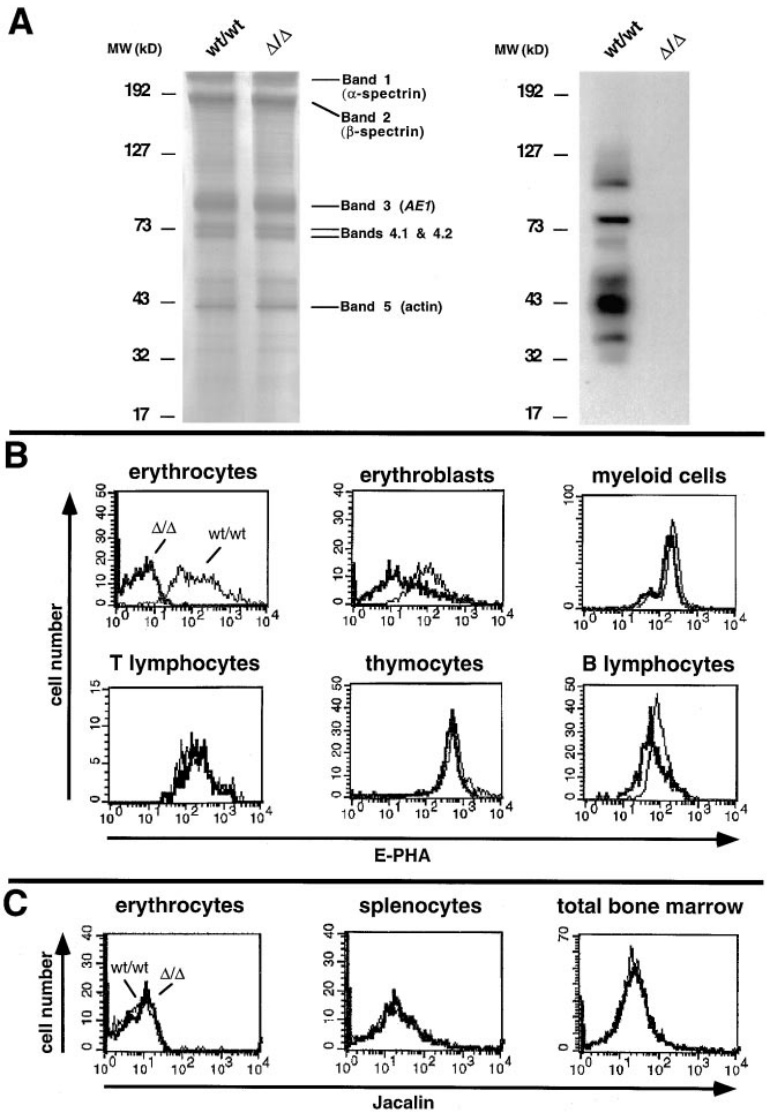

Figure 5. Erythrocyte Membranes from αM-II-Null Mice Are Uniquely Deficient in Complex N-Glycans

(A, left panel) Major membrane and cytoskeletal components (erythrocyte "bands") were visualized by SDS-PAGE and Coomassie blue staining. Using an alternate electrophoretic method (Fairbanks et al., 1971), no changes were observed in ankyrin expression in αM-II-null samples (data not shown).

(A, right panel) Complex N-glycans are visualized in wild-type samples but are absent from mutant membranes.

(B) E-PHA-biotin and antibody-FITC conjugates specific for cell lineage and differentiation antigens were combined in two-channel fluorescent-activated cell sorting analyses (Experimental Procedures). Among hematopoietic cell types from αM-II-null mice, circulating erythrocytes (RBCs) lacked significant binding to E-PHA, and bone marrow erythroblasts variably displayed a reduced level of E-PHA binding.

(C) O-glycosylation as judged by Jacalin lectin binding was unaltered among the indicated cell types as wild-type and mutant FACS profiles overlapped.

exposed mannose residues at the cell surface in αM-II-deficient mice may also target erythrocytes for interaction with the mannose-receptor and macrophage engulfment within the spleen. In comparison with human erythrocyte glycoproteins known to contain complex N-linked oligosaccharides, mouse versions appear distinct and do not produce complex N-linked oligosaccharides on band 3. However, human and mouse glycophorin A may normally contain high levels of sialic acid on complex N-linked oligosaccharide termini (Angel et al., 1991). Absence of complex N-linked oligosaccharides may yield glycoproteins that are hypomorphic, dysfunctional, or destabilized and perhaps absent from the membrane. Multiple targets as potential phenotypic effectors commonly arise in studies of enzyme deficiencies. Likewise, identification of altered glycoproteins in αM-II-deficient erythrocytes will be relevant in defining the inductive events that precipitate dyserythropoietic anemia.

The human CDAs comprise a heterogenic group of three clinically defined anemias for which the genetic bases are yet unresolved (Beutler, 1995). αM-II-deficient mice exhibit significant phenotypic similarities with human CDA type II. Splenomegaly with increased marrow erythroblasts are common to both human and mouse syndromes, as are the variable severity of anemia, anisocytosis, and increased i-antigen levels. In CDA type II, the increase in i-antigen levels occurs on glycolipids. In analyses of erythrocyte glycolipid structures from αM-II-null mice, we observed a similar increase in those containing the endo-β-galactosidase sensitive i-antigen polylactosamine (data not shown). Since normal mouse erythrocytes display low levels of I- and i-antigens, i-antigen induction may reflect a decreased erythroid maturation time common to the dyserythropoietic marrow (Beutler, 1995). Glycosylation abnormalities in CDA type II otherwise occur in a manner that would be expected for aberrant N-glycan production with deficiency of αM-II, GlcNAc-TII, or a galactosyltransferase (Fukuda et al., 1987, 1989, 1990). Nevertheless, lack of familial genetic data and the presence of normal αM-II levels in all but one case reported thus far calls into question the

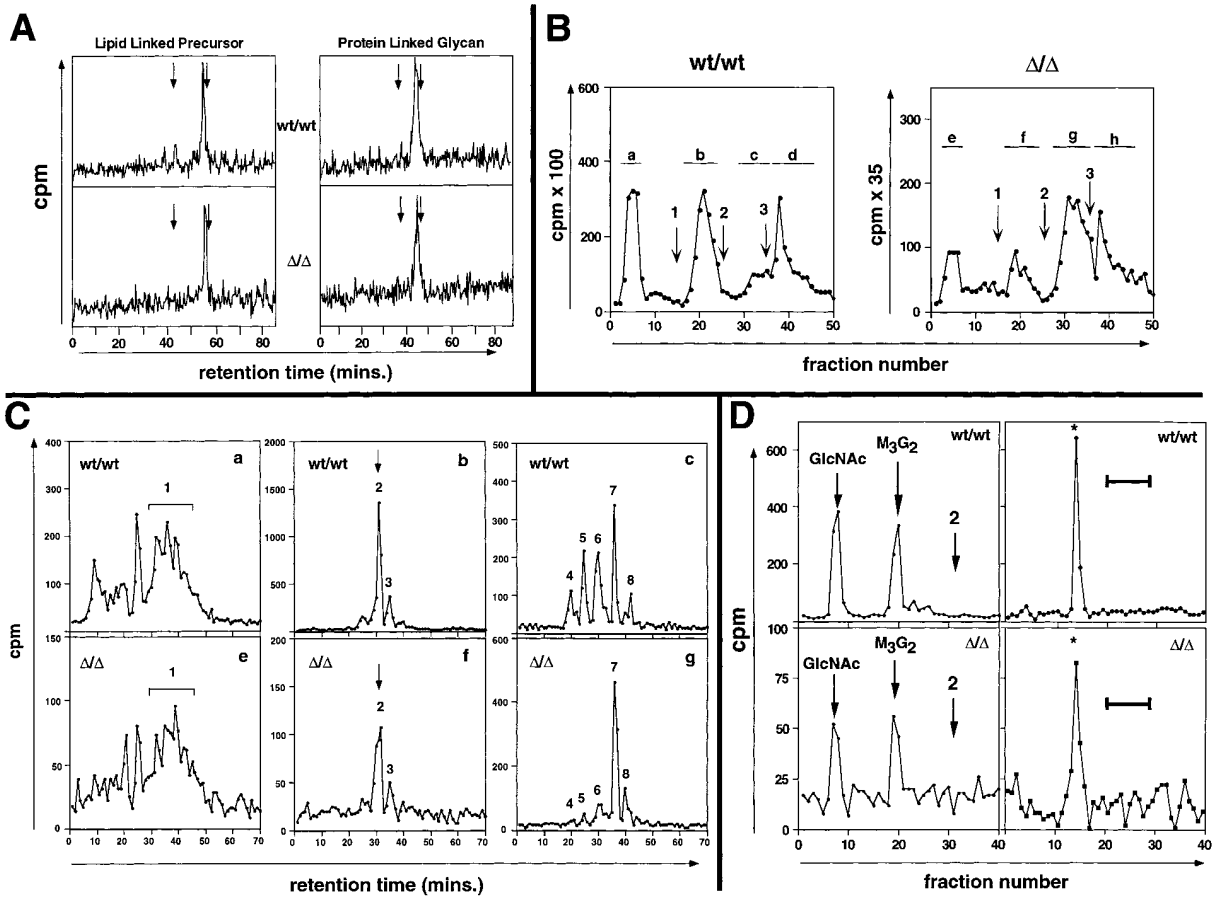

Figure 6. Complex N-linked Oligosaccharides Produced in the Absence of αM-II

Oligosaccharides from splenocytes and primary fibroblasts were metabolically labeled and analyzed as described (Experimental Procedures).

(A) Fibroblast-derived lipid-linked precursor and nascent protein-linked N-glycans migrated with a normal-sized oligosaccharide precursor (right arrow) and not with a truncated precursor produced in the absence of Dol-P-Man (left arrow).

(B) ConA-affinity chromatographic separation of splenocyte N-linked oligosaccharides. Tetra- and triantennary complex N-linked oligosaccharides are enriched in flow-through fractions and found in both genotypic samples (a and e). Biantennary complex N-linked oligosaccharides eluting with 10 mM α-methyl-glucoside (1) were present but in reduced abundance in αM-II-null cells (b and f). Hybrid N-linked oligosaccharides eluting with 10 mM α-methyl-mannoside (2) were increased in αM-II-deficient cells (compare c and g). The proportion of high mannose oligosaccharides eluting with 0.2 M α-methyl-mannoside appeared unchanged (d and h). Similar results were obtained with primary fibroblasts (data not shown).

(C) HPLC resolution of splenocyte oligosaccharides separated by ConA-Sepharose affinity chromatography. Hybrid N-glycan (peak 7) was increased in αM-II-deficient splenocytes and was defined by exoglycosidase treatment as the structure: Galβ1–4GlcNAcβ1–2Manα1–3[Manα1–3(Manα1–6)Man α1–6]Manβ1–4Glc NAcβ1–2GlcNAc (data not shown). HPLC fractions were also collected corresponding to tetra- and triantennary (a, e; peak 1) and biantennary (b, f; peak 2) N-linked oligosaccharides. The N-linked oligosaccharide in peak 2 comigrated with a bianntenary complex oligosaccharide standard (arrow).

(D, left) Sequential exoglycosidase treatment was applied to the N-linked oligosaccharide in HPLC peak 2. Studies confirmed the standard biantennary structure including the trimannosyl core (M_3G_2: Manα1–3[Manα1–6]Manβ1–4GlcNAcβ1–4GlcNAc). Position of released, labeled GlcNAc is indicated as is the original mobility of peak 2.

(D, right) Identical results were obtained using primary fibroblasts. Brackets denote elution position of undigested oligosaccharides derived from 10 mM α-methyl glucoside-eluted ConA-Sepharose fractions (data not shown). Elution position of M_3G_2 standard is indicated (asterisk).

role of αM-II mutation in most instances of human CDA type II.

Significant differences appear between CDA type II and mouse αM-II deficiency. No evidence for multinucleated erythroblasts could be found in the αM-II-deficient mouse bone marrow, although these are commonly found in marrow from CDA type II patients. Moreover, electron microscopic analyses did not detect the "double membrane" effect that may reflect incomplete loss of the endoplasmic reticulum in some CDA type II erythrocytes (Fukuda, 1993). Neither was there hepatomegaly, liver cirrhosis, or hemosiderosis. It is possible that these phenotypic variations reflect species-specific glycosylation functions, or perhaps the genetic heterogeneity of human CDA type II. Whatever the reasons for the reported differences, our results indicate that loss of αM-II gene function is one genetic basis for congenital dyserythropoietic anemia.

Table 1. Percent Abundance of N-linked Oligosaccharide Structures in Splenocytes and Primary Fibroblasts from Wild-Type and αM-II-Null Mice

N-Linked Oligosaccharides	Wild-Type	αM-II-Null
Splenocytes		
High mannose	31.9	26.1
Hybrid	17.4	51.1
Complex	50.7	22.8
Primary Fibroblasts		
High mannose	47.5	48.5
Hybrid	12.5	35.0
Complex	40.0	16.5

Amount of radiolabeled N-linked oligosaccharides residing in ConA-Sepharose fractions (Figure 6B) calculated as a percentage of total cpm.

The Alternate Pathway to Complex N-Glycan Production in Normal and Disease Physiology

The alternate pathway in complex N-glycan biosynthesis provides a mechanism to compensate for the absence of αM-II. Nevertheless, erythroid cells cannot compensate for αM-II deficiency, and this appears to explain the cell-type specificity of the phenotype observed. Why the alternate pathway does not seemingly function in erythroid cells requires additional investigation. It is possible that αM-III is simply not expressed in erythroblasts or exists in an inactivated form. Alternatively, αM-III may be relatively labile, with loss of cofactors occurring early in erythrocyte maturation and prior to enucleation. Characterization of the gene(s) responsible for encoding αM-III activity is needed to address these issues. In this regard, recent analyses of a human gene product termed αM-IIx (Misago et al., 1995) have revealed that the encoded αM enzyme is located in the *cis* Golgi and acts on $Man_5GlcNAc_2-$ to produce $Man_3GlcNAc_2-$ (M. N. F., unpublished data). Whether human αM-IIx is the homolog of the gene encoding αM-III activity in the mouse is unclear, as αM-IIx activity does not appear to be dependent upon Co^{2+}. Multiple αM enzymes that process $Man_5GlcNAc_2$-Asn may exist; alternatively, rodent and human αM-IIx may be orthologous with different enzymatic properties arising from significant structural divergence.

Among nonerythroid cells studied in αM-II-deficient mice, these harbored approximately 50% of normal complex N-glycan levels, suggesting that αM-III activity functions both initially and at high efficiency in catalyzing the conversion to complex N-glycans. It is possible that GlcNAc-TI competes with αM-III for the $Man_5GlcNAc_2$-Asn N-glycan, perhaps leading to a difference in the repertoire of N-glycans on some αM-II-deficient nonerythroid cells. αM-II may also be compensatory in some situations of αM-III deficiency. Such an apparent redundancy is actually advantageous, since vertebrates lacking complex N-glycans from GlcNAc-TII-deficiency are severely impaired with neurologic and developmental complications, leading to lethality prior to reproductive age (Jaeken et al., 1994; Tan et al., 1996; R. Campbell and J. M., unpublished data). The αM-II-deficient mouse has provided intriguing questions to be considered in revealing an alternate pathway in N-glycan biosynthesis, without which erythroid complex N-glycan production fails and dyserythropoietic anemia ensues.

Experimental Procedures

Production of Targeted Embryonic Stem Cell Lines and αM-II-Deficient Mice

The mouse *αM-II* cDNA (Moremen and Robbins, 1991) was used to isolate a 17 kb mouse genomic *αM-II* clone from a 129/SvJ library (Stratagene) by cross-hybridization as previously described (Priatel, 1997). Sequence analysis revealed a single *αM-II* exon corresponding to amino acids 236–258. Construction of an αM-II gene-targeting vector was accomplished using the pflox vector (Figure 2A). The linearized αM-II-targeting DNA construct (10 μg) was introduced into R1 ES cells via electroporation, prior to selection with 150 μg/ml of active G418 (Life Technologies) for 10 days on gelatin-coated tissue culture dishes as described (Priatel et al., 1997). PCR positive clones were analyzed using an αM-II genomic probe consisting of a 1.1 kb SmaI-XbaI fragment as denoted (Figure 2A). The *loxP* probe consisted of two *loxP* sites separated by plasmid multiple-cloning sites (Hennet et al., 1995). ES cell clones bearing a targeted *αM-II* allele were electroporated with 10 μg of supercoiled Cre expression plasmid pCre-Hygro and plated at low dilution. After 4 days, ganciclovir (2×10^6 M) was added, and, 5 days later, resistant subclones were analyzed by Southern blotting. Chimeric mice were produced by microinjection of 8–10 clonal ES cells into 3.5 day C57BL/6 blastocyst-stage embryos, followed by implantation as described (Metzler et al., 1994). The altered *αM-II* allele was crossed into the C57BL/6 background for the production of heterozygous αM-II mutant mice and bred with C57BL/6 mates for at least three to five generations. Homozygous $\alpha M\text{-}II^{\Delta}$ mice and littermate control animals used in this study were generated by crosses involving these $\alpha M\text{-}II^{\Delta}$ heterozygotes.

αM-II and αM-III Enzymatic Analyses

Mouse tissues were homogenized in 1–2 ml of extraction buffer (2% Triton X-100, 0.5 M NaCl, 20 mM potassium phosphate [pH 7.5]),

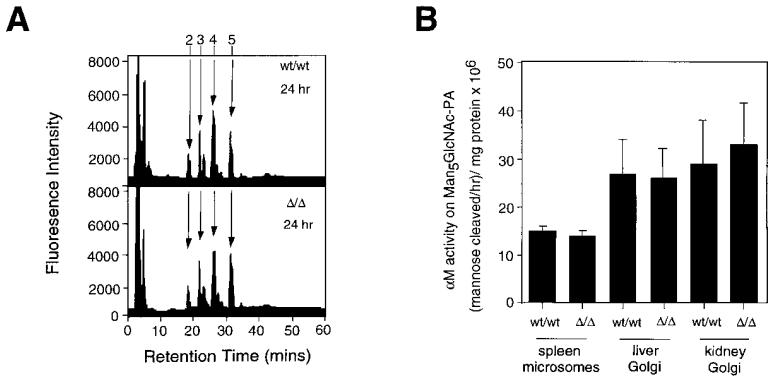

Figure 7. Presence of a Distinct αM Activity Processing $Man_5GlcNAc$-PA in Golgi and Microsomal Preparations from Normal and αM-II-Deficient Cells

(A) Splenocyte microsomal fractions exhibited an αM-processing activity toward $Man_5GlcNAc$-PA (5) yielding $Man_4GlcNAc$-PA (4), $Man_3GlcNAc$-PA (3), and $Man_2GlcNAc$-PA (2) by 24 hr (Experimental Procedures).

(B) This αM activity was also observed at similar levels in Golgi fractions of liver and kidney from wild-type and αM-II-null mice.

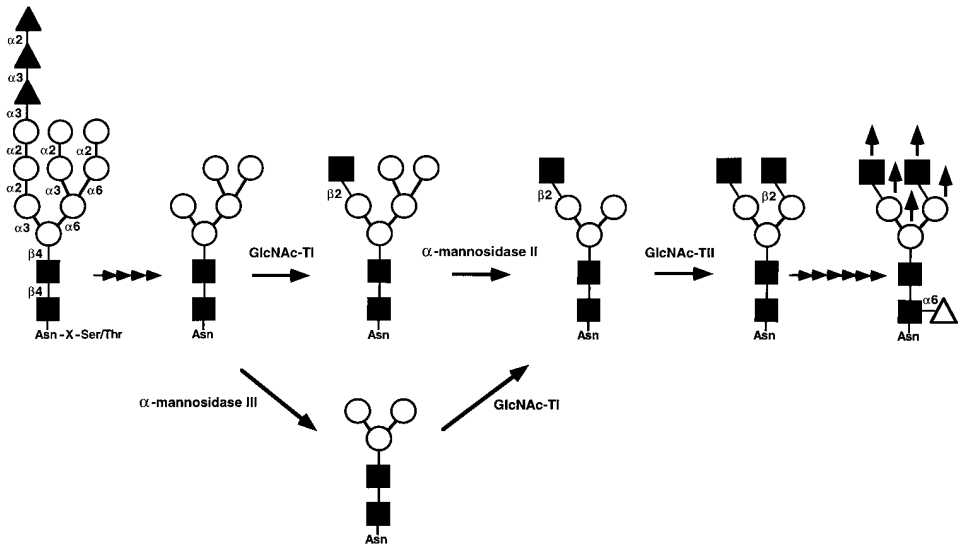

Figure 8. Proposed Revision in the Biosynthetic Pathway of Complex Asparagine-Linked Oligosaccharide Production

The αM-III designation is a tentative nomenclature based upon the differences in αM-I and αM-II activities previously defined within the N-glycan processing pathway. The Co^{2+}-activated αM previously described (Bonay and Hughes, 1991; Bonay et al., 1992) is a likely candidate for the αM-III activity detected herein among Golgi and microsomal fractions, although it is not presently possible to rule out the existence of multiple isozymes.

centrifuged at 16,000 × g for 5 min, and assayed for αM activity with pNP-Man and $GlcNAc_1Man_5GlcNAc_1$-PA substrates as total cellular supernatants, or as immunoprecipitates using 2 μl anti-Man II antiserum (Moremen et al., 1991; Liao et al., 1996). Microsomes and Golgi membranes were prepared as previously described (Moremen and Touster, 1986) and stored at −20°C prior to analyses with $GlcNAc_1Man_5GlcNAc$-PA and $Man_5GlcNAc$-PA as substrates in 100 mM NaAc (pH 5.6) and 100 mM N-acetylglucosamine, or in 100 mM NaAc (pH 6.5), 100 mM N-acetylglucosamine, and 1 mM $CoCl_2$ (Bonay and Hughes, 1991; Liao et al., 1996; Merkle et al., 1997). In all assays, sensitivity to inhibition by swainsonine was tested using a 10 μM final concentration of the alkaloid. $GlcNAc_1Man_5GlcNAc$ and $Man_5GlcNAc$ were kindly provided by Dr. Jeremy Carver (University of Toronto) and by Dr. Annette Herscovics (McGill Cancer Center, Montreal), respectively.

Hematology and Histology

Mice were anesthetized with methoxyfluorane, and blood was collected from the tail vein into EDTA-coated polypropylene tubes (Becton Dickinson). A CELL-DYN 3500 programmed for normal mouse parameters was used to assess cellular size, volume, and morphology involving red blood cells, leukocytes, and platelets as described (Priatel et al., 1997). Parallel blood smears were performed and stained with Wright-Giemsa solution or new methylene blue (for reticulocyte counts) for manual analyses. Osmotic fragility measured percent hemolysis by OD_{540} in response to indicated NaCl concentrations. Levels of RBC Ii-antigens were defined with antisera previously described (Feizi et al., 1979; Hakomori et al., 1981). Anti-I or anti-i antisera was serially diluted with PBS in a round-bottom microtiter plate. RBCs from wild-type or αM-II-null mice were added to each well and incubated on ice for 4 hr, after which the presence or absence of hemagglutination was assessed.

Histologic studies were performed on paraffin or frozen tissue sections by methods previously described (Campbell et al., 1995). Staining and lectin reagents included hematoxylin/eosin, Oil red O, Sudan black, iron (Perls reaction), and lectins E-PHA, ConA, Jacalin, and L-PHA (Vector Laboratories).

Flow Cytometric Analyses

Single cell suspensions were isolated from mice and subjected to hypotonic lysis of red blood cells, using ammonium chloride when indicated. Cells were counted using a hemocytometer, and 500,000 were labeled in a final volume of 100 μl with either fluorescein (FITC)-conjugated E-PHA (1 μg/ml) or Jacalin-FITC (0.5 μg/ml) (Vector Laboratories). Subsequently, cells were incubated with 1 μl of indicated antibody for 10 min. All incubations and washes were performed on ice in FACS buffer (2% FCS in PBS). Cells were analyzed using a FACScan Flow Cytometer and CellQuest Software (Becton Dickinson, Mountain View, CA) as previously described (Hennet et al., 1995). Lineage-specific antibodies included erythroid phycoerythrin (PE)-conjugated TER-119, myeloid anti-Ly-6G-PE (Gr-1, RB6-8C5), B lymphoid anti-B220-PE (RA3-6B2), and T lymphoid anti-CD3-PE (145-2C11) (Pharmingen).

Asparagine-Linked Oligosaccharide Characterization

Tail biopsy-derived primary fibroblasts were labeled in 5.5 mM glucose DMEM with 20 μCi/ml [2-^{3}H]mannose for 24 hr. Preparation of ^{3}H-mannose-labeled oligosaccharides hydrolyzed from precursor lipid or from protein by PNGaseF digestion was as described (Sampath et al., 1992; Panneerselvam and Freeze, 1996). Splenocytes were labeled with 20 μCi/ml ^{3}H-glucosamine for 24 hr in 98% glucose-deficient medium with 2% normal RPMI 1640 and 10% dialyzed fetal calf serum. Glycoproteins were extracted with 0.5% Triton X-100 in 0.1 M Tris-HCl (pH 6.8) containing 0.1 M NaCl, 0.3 M sucrose, 3 mM $MgCl_2$, 1 mM PMSF, and digested with PNGaseF. Released N-linked oligosaccharides were reduced to alditols with 1 M $NaBH_4$ and applied to 1 × 4 cm ConA Sepharose columns equilibrated in 10 mM Tris-HCl (pH 7.5). Oligosaccharides were eluted sequentially with 10 mM α-methyl glucoside, 10 mM α-methyl mannoside, and 0.2 M α-methyl mannoside. Radioactivity was monitored in 1 ml fractions produced. Oligosaccharide-alditols resolved by ConA-Sepharose chromatography were desialyated by mild acid hydrolysis and analyzed by ion suppression amine adsorption (ISAA)/HPLC using a Varian MicroPak AX-5 column. Standard high mannose-type oligosaccharides, M9, M8, M7, M6, M5, and a biantennary complex type oligosaccharide, NA2 (Galβ1–4GlcNAcβ1–2Manα1–3[Galβ1–4GlcNAcβ1–2Manα1–6]Manβ1–4GlcNAcβ1–4GlcNAcOH), were purchased from Oxford Glycosystems (Oxford, U. K.). M_3G_2 (Manα1–3[Manα1–6]Manβ1–4GlcNAcβ1–4GlcNAcOH) was prepared from NA2 by exoglycosidase digestion. ConA-Sepharose- and HPLC-separated oligosaccharides were exoglycosidase treated as described (Merkle and Cummings, 1987).

RBC Protein, Glycoprotein, and Glycolipid Analyses

Erythrocyte membranes were prepared and analyzed by SDS-PAGE as previously described (Fairbanks et al., 1971; Fukuda et al., 1984). In other studies, intact erythrocytes were labeled with $NaB[^3H]_4$ to incorporate tritium on nonreducing terminal galactose and N-acetylgalactosamine residues in glycoproteins and glycolipids (Fukuda et al., 1979) prior to SDS-PAGE and autoradiography. For lectin blotting, SDS-PAGE-resolved proteins were transferred onto nitrocellulose and incubated in PBS-T (PBS containing 0.05% Tween-20) supplemented with 3% BSA for 4 hr. Blots were subsequently incubated with 2 μg/ml biotinylated E-PHA (Vector) for 1 hr at 25°C, washed three times in PBS-T, and incubated with avidin-horseradish peroxidase for 30 min prior to development, using ECL as described (Amersham). For glycolipid analyses, erythrocyte glycolipids were extracted from $NaB[^3H]_4$-labeled membranes with chloroform methanol (2:1, v/v), analyzed by thin-layer chromatography on an HPTLC silica gel plate (Baker, Si-HPF plate), and developed in a solvent composed of chloroform, methanol, and water (60:35:8, v/v). Radioactive erythrocyte glycolipids were visualized by fluorography and resolved in relation to the migration of glycolipid standards.

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